Eur J Vasc Endovasc Surg **26**, 205–210 (2003)
doi:10.1053/ejvs.2002.1874, available online at http://www.sciencedirect.com on SCIENCE DIRECT®

Genetic Linkage of Candidate Genes in Families with Abdominal Aortic Aneurysms?

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Objectives: *to examine possible involvement of several candidate genes in the aetiology of familial abdominal aortic aneurysm (AAA).*
Design: *after reviewing the literature on the genetics of familial AAA, betaine homocysteine methyltransferase (BHMT), collagen type Iα2 (COL1A2) and cathepsin H (CTSH), were selected as potential candidate genes, which influence structure, strength, elasticity and mechanical resistance of the aortic wall.*
Materials: *forty-eight families with 110 family members and AAA were included in the affected sib-pair analysis. One large family of three generations was analysed separately because in this family also other clinical symptoms were involved.*
Methods: *genetic linkage analysis was performed with DNA markers in the region of BHMT, COL1A2 and CTSH.*
Results: *In the overall sib-pair analysis, the LOD scores for BHMT, COL1A2 and CTSH were 0.7, 0.2 and −0.7, whereas in the large family these numbers were −0.6, −2.2 and −2.7, respectively.*
Conclusions*: none of the candidate genes selected showed a suggestive linkage with AAA. Exclusion of the COL1A2 and CTSH genes was possible in the large family that was analysed separately.*

Key Words: Familial abdominal aortic aneurysm; Genetic aetiology; Candidate genes; Linkage analysis.

Introduction

Despite evidence indicating a familial predisposition to abdominal aortic aneurysm (AAA),[1–4] it appears difficult to identify the specific genes involved. Recently, we have reviewed the genetic background of familial AAA.[5] Guo *et al*.[6] performed linkage analysis in patients with familial thoracic aortic aneurysms (TAA). A major locus for familial TAA mapped to chromosome 5q13-14. The betaine-homocysteine methyltransferase (BHMT) gene is localised here and might be a possible candidate gene. BHMT catalyses the conversion of betaine and homocysteine to dimethylglycine and methionine. Hyperhomocysteinaemia is a risk factor for arterial occlusive disease, but probably also for aneurysmal disease.[7–10] Vouyouka *et al*.[11] demonstrated a significant weakening of the aortic wall in collagen type Iα2 (COL1A2) knock-out mice. The integral role of COL1A2 in the biomechanical and functional properties of the aorta might help to elucidate the role of collagen in the development of aneurysmal aortic disease. Tung *et al*.[12] found a 30-fold upregulation of cathepsin H (CTSH) in the aneurysmal wall of the abdominal aorta, using a commercially available membrane-based complementary DNA expression array. Increased activity of cathepsin H, which is a cysteine- dependent protease, can mediate extensive matrix breakdown, thereby negatively influencing the elasticity and mechanical resistance of the aortic wall. The aim of this study was to perform genetic linkage analysis of betaine homocysteine methyltransferase, collagen type Iα2 and cathepsin H in patients with familial AAA.

Materials and Methods

Subjects and family collection

Computerised records containing diagnostic codes for the years 1996–2001 of 12 hospitals, with a department of vascular surgery, in the north-west region of the

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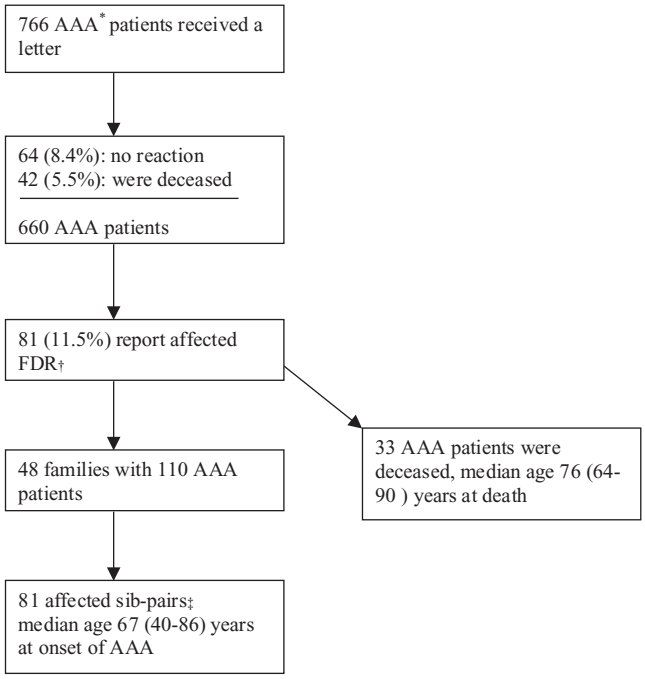
Netherlands were searched for patients diagnosed with, infrarenal abdominal aortic aneurysm with or without rupture (ICD codes 4413(-02), 4414(-02)). An AAA was defined as an aortic diameter greater than 3 cm in the infrarenal portion of the aorta, or a diameter of 50% greater than the normal suprarenal aorta.[13] A total of 766 AAA patients complied with these diagnostic criteria. These patients received a letter, asking for information about their family history. A positive family history for AAA was defined as one or more first-degree relatives (FDRs) with an AAA. Patients with the Ehlers Danlos syndrome type IV were excluded. There was a 91.6% response rate after one recall letter and 81 persons claimed to have one or more FDRs with an AAA. Forty eight persons, 44 males and four females (index cases), with 53 brothers and nine sisters were able to participate in the study. One AAA patient with one affected FDR is called an affected sib-pair (Flow diagram and Table 1). One large family of three generations was analysed separately because of additional clinical symptoms other than AAA, including bruises, skin hyperextensibility and joint hypermobility (Fig. 1). Previously, linkage to collagen type III and V, fibrillin 1 and elastin had been excluded in this family. Subsequently, the medical records for the index patients and their relatives were

Table 1. Number of affected relatives and the number of sib-pairs in AAA families include in the study.

Affecteds *N*	Families *N*	Sib-pairs* *N*
5	1	10
4	2	12
3	7	21
2	38	38
Total	48	81

* Number of sib-pairs per family: 2 affecteds = 1, 3 affecteds = 3, 4 affecteds = 6, 5 affecteds = 10 sib-pair(s).



Flow diagram. *An AAA was defined as an aortic diameter greater than 3 cm in the infrarenal portion of the aorta, or a diameter of 50% greater than the normal suprarenal aorta.[13] †The patients with one or more first-degree relative (FDR). ‡An AAA patient with one affected FDR is called an affected sib-pair. See Table 1.

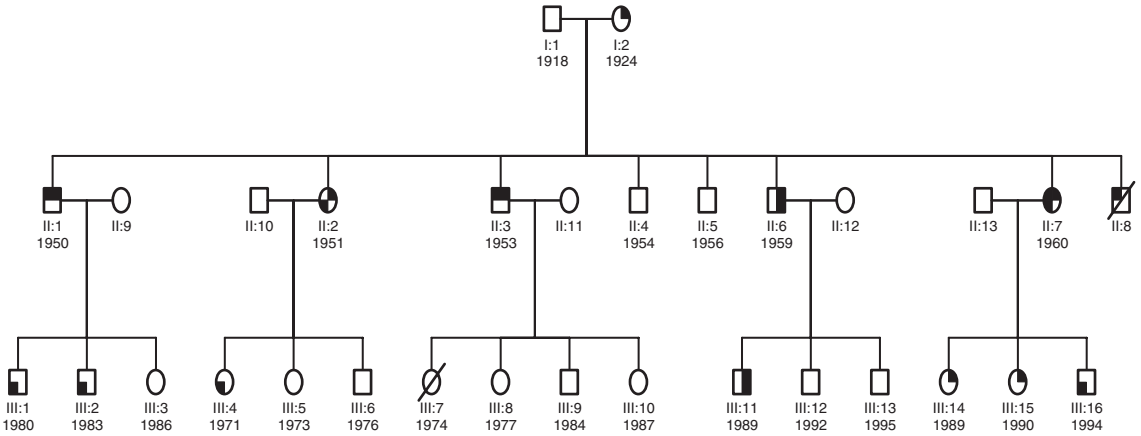


Fig. 1. The figure shows the family tree of the large family with abdominal aortic aneurysms (AAA) and other symptoms. Filled squares and circles of the upper left indicates those in whom AAA was diagnosed, filled squares and circles of the upper right indicates those in whom bruises was diagnosed, filled squares and circles of the lower left indicates those in whom joint hypermobility was diagnosed, filled squares and circles of the lower right indicates those in whom skin hyperextensibility was diagnosed. All other individuals are not known to be affected and are treated as unknown in the analysis. I–III = different generations and year of birth. Individual II-8 died of a ruptured AAA at the age of 30 years. Addition symptoms: II-3 pulmonary aneurysm and tricuspid valve regurgitation; II-2 varicosis.

reviewed and the diagnoses verified. We invited the index patients and their relatives for recording of the medical and family history and the collection of blood samples for DNA analyses. These studies were approved by the local ethics committee and all patients and their family members gave informed consent.

Material

DNA was extracted from peripheral blood, using DNAzol reagent (Invitrogen, Molecular Research Center, Inc., Cincinnati, OH, U.S.A.). Variable number tandem repeats (VNTRs) markers analysed in the BHMT region were D5S646 and D5S2029, for COL1A2 D7S1799, D7S527 and D7S657 and for CTSH D15S153, D15S653, D15S973 and D15S1023. Fluorescent labelled primers were customade by Invitrogen. Average heterozygosity of the markers is present in 66–87% of CEPH families. For 25 µl polymerase chain reaction (PCR) mix we used 100 ng DNA, 1.5 mM $MgCl_2$, 200 µM dNTP's and 10 picomoles primers. A control sample was derived of CEPH1347-02 DNA (www.ceph.fr). The PCR conditions (Perkin Elmer 9600) used were 94 °C for 2 min to activate the platinum Tag-polymerase, 35 cycles of denaturation at 94 °C for 1 min, annealing at 59–57–55 °C for respectively 5–5–25 cycles and elongation at 72 °C for 1 min. The pooled products were supplemented with an internal size standard and fragment analysis was performed on an ABI PRISM 310 Genetic Analyser (Applied Biosystems, Torrance, CA, U.S.A.).

Statistical analysis

Linkage analysis is most often used to localise a disease gene by virtue of its linkage to a genetic marker locus on the gene map. Linkage of a disease phenotype and a marker means that two are located closely on the same physical piece of DNA. The closer two loci occur on a piece of DNA, the less frequent crossovers are and the more tightly the two are linked. The linkage between the disease phenotype and the marker can be expressed as a distance in genetic map units (centimorgan; cM). Linkage can be established whenever the phenotype and genotype of related individuals is analysed, therefore linkage can be established by analysing large families or by analysing pairs of siblings.[14,15] For diseases with a known Mendelian mode of inheritance, the appropriate technique is the parametric LOD score (maximum likelihood) approach. The non-parametric affected sib-pair approach, does not require any assumption on mode of inheritance and only looks for deviations in the inheritance of marker alleles from parent to affected offspring. Many non-parametric approaches estimate identity by descent (IBD) allele sharing by maximum likelihood and then present results in terms of LOD scores. Both approaches may be applied with the aid of computer programs. This typically results in a "LOD score curve" along the chromosome whose maximum identifies the estimated position of the disease locus and whose magnitude is indicative of the strength of linkage.[16]

Affected sib-pair analyses were performed using the Allegro version 1.1 computer program for multipoint genetic linkage analysis.[17] The null-hypothesis of this analysis, assuming no linkage between the disease and tested markers, is that 25% of siblings share zero alleles, 50% share one allele and 25% share two alleles for each marker. Positive linkage between a marker and disease, irrespective of the mode of inheritance, will be reflected by a deviation from these proportions with an excess of allele sharing. Results are given as non-parametric multipoint LOD scores. The LOD score in allele sharing analyses is the base 10 logarithm of the ratio between the likelihood that observed excess allele sharing that is based on linkage between a marker and the disease and the likelihood that the excess sharing occurred by chance. A LOD score above 3.0 is considered significant for linkage.[18] The term "multipoint" means that information of all markers for one candidate gene was used simultaneously. In sib-pair analyses a LOD score lower than -2 is considered significant evidence for exclusion of linkage.[19] The Mega 2 version 2.3 data conversion program and SimWalk2 version 2.82 statistical genetics computer program for Windows were used to analyse the large family.[20,21] The linkage analysis was performed parametric, assuming an autosomal dominant inheritance with reduced penetrance as the genetic model (Fig. 1). The prevalence of patients with the same clinical symptoms in the whole population was assumed to be 0.01% and the penetrance of mutant genes was set at 80% with dominant inheritance. All individuals not known to be affected were classified as "unknown".

Results

Clinical characteristics of classical risk factors like smoking habits, the presence of hypertension (WHO criteria), diabetes mellitus and hypercholesterolaemia (all investigated by a physician), and atherosclerotic manifestations such as peripheral, coronary and

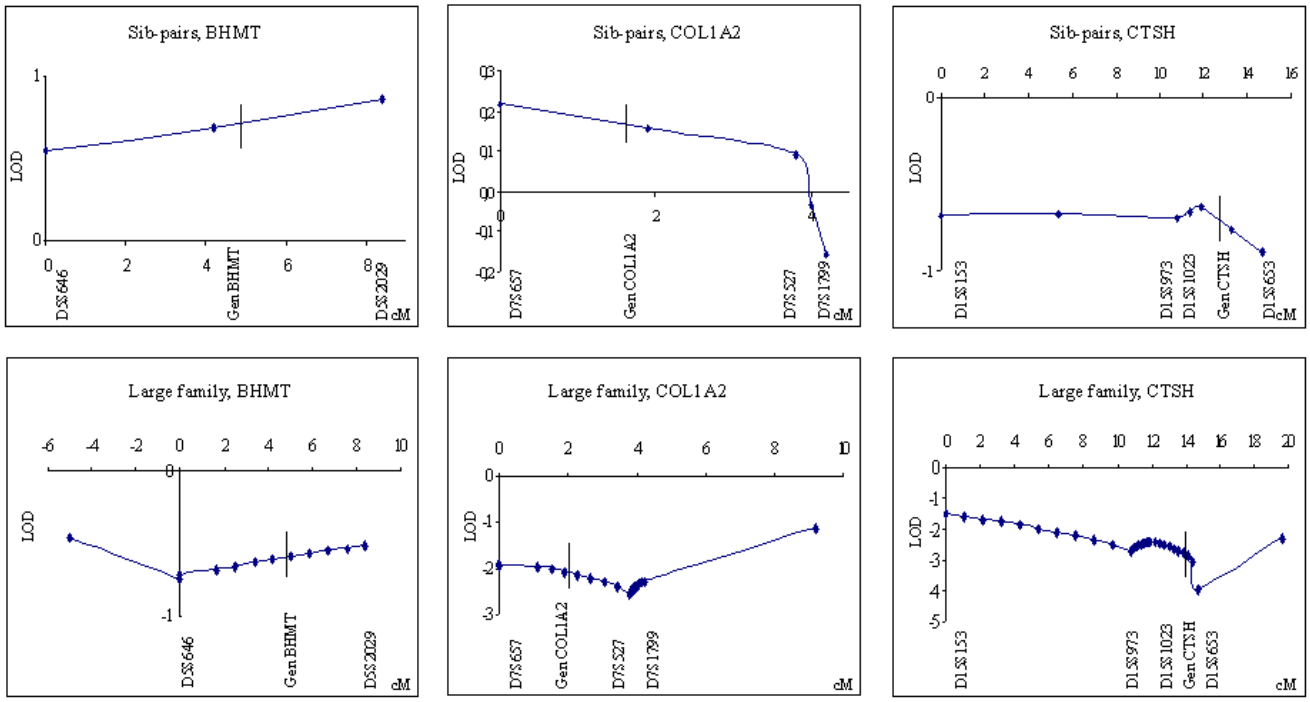


Fig. 2. Results of linkage analysis of DNA markers in the region of betaine homocysteine methyltransferase (BHMT), collagen type1α2 (COL1A2) and cathepsin H (CTSH) in siblings with abdominal aortic aneurysms (AAA) and in a large family of three generations with AAA and other symptoms (see Fig. 1). Each box represents the results of the candidate gene, the y-axis depicts the multipoint LOD score, the x-axis the cM distance of the markers used. The vertical line is an estimation of the position of the candidate gene compared to the DNA markers.

cerebral arterial occlusive disease were not significantly different compared with AAA patients without familial AAA (unpublished data), as earlier described.[22] Results of the sib-pair and large family genetic linkage analyses are shown in Figure 2. The maximal simulated LOD scores calculated, assuming the presence of linkage, for the siblings and large family were 8.6 and 4.4, respectively. We estimated the position of the candidate gene compared to the position of the DNA markers in the genomic DNA sequence (www.ncbi.nlm.gov.entrez/nucleotide). The positions are indicated with a vertical line in Figure 2. The exact relative positions in cM are not known, but it is assumed that no large differences in recombination frequencies exist within the small regions between the markers. The LOD scores at the position of the candidate genes were in the sib-pair analyses were 0.7, 0.2 and −0.7 at the locations of the BHMT, COL1A2 and CTSH genes, respectively. In the large family, the LOD scores were −0.6, −2.2 and −2.7, respectively. In the large family, the genes for COL1A2 and CTSH could be excluded as a possible candidate gene. None of the other tested genes could be proven or excluded as a possible candidate because of the low positive and negative LOD scores.

Discussion

Genetic linkage analysis is a technique for measuring the genetic distance between two loci on a chromosome. Alleles at loci in close proximity with each other will be co-inherited as a "package" (haplotype) from parents to offspring.[16] Particularly, linkage in a large family, with several generations and large offspring, it will be expected that the same gene(s) is/are responsible for the disease. Linkage analysis of regions containing the genes BHMT, COL1A2 and CTSH was chosen after a systematic research of the literature.[5]We found no evidence of linkage of the candidate genes BHMT, COL1A2 and CTSH for familial AAA. None of the regions attained a LOD score higher than 3.0. Linkage was excluded in the large family for the COL1A2 and CTSH genes. Linkage could not be excluded for the other regions with low positive or negative LOD scores listed above and none of the results showed a suggestive linkage with AAA. BHMT, next to methionine synthase, serves as a facilitator of methyl group donation for remethylation of homocysteine into methionine and reduced functioning of BHMT could theoretically result in elevated homocysteine levels. The actual events that cause

aneurysmal degeneration and their relation to atherosclerosis are unsolved. Arteriosclerosis is predominantly a disease of the intima whereas AAA involves a transmural degenerative process. A possible explanation can be found in the induction of elastolysis by homocysteine in arterial media through the activation of matrix metalloproteinase-2 (MMP-2), demonstrated in an animal model.[23] Although elevated homocysteine levels have been associated with arteriosclerosis and aneurysmal disease[8,10,24] our results provide no evidence for linkage of BHMT with AAA. Seventy percent of the extracellular matrix of the abdominal aortic wall consists of collagen, providing the integrity to withstand the outward forces exerted by arterial pressure. More than 150 different mutations have been reported in the genes for procollagen type I (COL1A1 and COL1A2). Mutations in COL1A2 cause heritable disorders of connective tissue, i.e. osteogenesis imperfecta type II (OI, brittle bone syndrome) and Ehlers Danlos Syndrome type VII (EDS VII, characterised by excessive joint hypermobility and skin abnormalities).[25] Mutations in procollagen type I do not have important influence on the vascular integrity that might contribute to aneurysmal degeneration. In contrast, Vouyaka *et al*.[11] showed in a mouse model that absence of collagen type 1α2 weakens the thoracic aorta. They demonstrated an integral role of collagen type 1α2 in the biomechanical and functional properties of the aorta. As mentioned above, the abdominal aorta consists of a higher percentage of collagen and alterations of collagen type 1α2 could have even more influence on the tensile strength of the aortic wall and in this way contribute to aneurysm formation. In the large family, but not in the sib-pair analysis, linkage to COL1A2 was excluded. The cathepsins comprise a group of intracellular proteases. Lysosomal proteases, i.e. cathepsins D, H and L, are involved in the degradation of structural proteins and were found to have higher activities in the aneurysmal wall and a mural thrombus than in the normal aortic wall.[12,26] The elastic and mechanical properties of the aorta can be negatively influenced by matrix breakdown due to cathepsin. Linkage to the region of CTSH was excluded in the large family, but not in the sib-pair group due to low negative LOD scores. Familial AAA is well documented, but the genetic aetiology remains unclear. Our study indicates no linkage of BHMT, COL1A2 or CTSH genes. Possible other candidate genes, which have influence on the degradation of the aortic wall, are the proteinases, i.e. matrix metalloproteinases, plasminogen activators, serine elastases and other cysteine proteases. The influence of inflammation and immune responses on the destruction of the aortic wall is also of subject for further investigation.[27] In the future we expect that genome-wide scans can identify susceptible loci. If loci are found, new candidate genes will be identified.

Acknowledgements

We thank the laboratory staff, J. Keek, E. Van den Akker, M. Grippeling and R.J. van Schooten, for excellent technical assistance. We thank the participating hospitals for including AAA patients and their family members (M. R. Sobotka, Onze Lieve Vrouwe Gasthuis, Amsterdam, P. J. van Aken and J. A. Lawson, Amstelveen Hospital, H. L. F. Brom and J. A. R. Coosemans, Elisabeth Gasthuis, Haarlem, B. Lether and J. H. Viersma, Spaarne Haarlem Hospital, G. J. M. Akkersdijk, Spaarne Heemstede Hospital, M. W. C. de Jonge, Westfries Gasthuis, Hoorn, H. A. van Dijk, P. J. van den Akker and R. W. Meijer, Medisch Centrum Alkmaar, J. J. M. Jutte, Gemini Hospital, Den Helder, G. A. Vos and Th. A. A. van den Broek, Waterland Hospital, Purmerend, R. A. Cohen and W. Mulder, Hospital de Heel, Zaandam, A. H. M. Dur and W. E. Tuinebreijer, Rode Kruis Hospital, Beverwijk). We also thank Drs E. van Vlijmen for linguistic reversion.

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Accepted 7 January 2003